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Re-evaluation of the Immunological Big Bang

Minireview

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Classically the immunological 'Big Bang' of adaptive immunity was believed to have resulted from the insertion of a transposon into an immunoglobulin superfamily gene member, initiating antigen receptor gene rearrangement via the RAG recombinase in an ancestor of jawed vertebrates. However, the discovery of a second, convergent adaptive immune system in jawless fish, focused on the so-called variable lymphocyte receptors (VLRs), was arguably the most exciting finding of the past decade in immunology and has drastically changed the view of immune origins. The recent report of a new lymphocyte lineage in lampreys, defined by the antigen receptor VLRC, suggests that there were three lymphocyte lineages in the common ancestor of jawless and jawed vertebrates that co-opted different antigen receptor supertypes. The transcriptional control of these lineages during development is predicted to be remarkably similar in both the jawless (agnathan) and jawed (gnathostome) vertebrates, suggesting that an early 'division of labor' among lymphocytes was a driving force in the emergence of adaptive immunity. The recent cartilaginous fish genome project suggests that most effector cytokines and chemokines were also present in these fish, and further studies of the lamprey and hagfish genomes will determine just how explosive the Big Bang actually was.

Introduction

Jawed vertebrates (gnathostomes) have an adaptive immune system grounded on their antigen receptors, immunoglobulins (Ig) or antibodies and T-cell receptors (TCRs), as well as the major histocompatibility complex (MHC). This system arose over a very short period of evolutionary time and has been christened the immunological 'Big Bang' [1]. Its rapid emergence was thought to be catalyzed by the horizontal transfer of the 'RAG transposon' [2] from an invading organism, which provided the enzymatic machinery and DNA signals to permit Ig/TCR diversity to be generated via somatic rearrangement. Over a short period of evolutionary time, antibodies and two types of TCRs were generated, followed closely by a complex network of regulation to enlarge the repertoire of immune function and to ensure protection against autoimmunity [3]. The system is so complex that it has been suggested (somewhat facetiously) that the emergence of adaptive immunity via the RAG transposon may not have been to our advantage, and thus we would have been better off with the preservation of an innate system in which there is no requirement for somatically generated tolerance mechanisms [4]!

Ten years ago, adaptive immunity was believed to be the exclusive domain of gnathostomes. The jawless vertebrates (agnathans), including the extant lamprey and hagfish, were known to have lymphocytes and even evidence of adaptive immunity; however, there was no trace of Ig, TCR or MHC molecules from studies of expressed sequence tags (ESTs) and a multitude of other attempts to find them [5]! Furthermore, we all agreed that the thymus — the primary lymphoid tissue that helps to define adaptive immunity in gnathostomes — was not present in these animals [6,7], nor was the secondary lymphoid tissue, the spleen, which is also present in all gnathostomes. This smug view, that agnathans lacked any distinctive molecular or tissue characteristics of adaptive immunity, was quashed by Pancer and Cooper, who in 2004 demonstrated that there was a second somatically generated antigen receptor family in lampreys [8], built upon an entirely different type of protein domain, the leucine-rich repeats (LRRs) (Figure 1). In this receptor, named the variable lymphocyte receptor (VLR), LRRs are encoded in small cassettes upstream and downstream of invariant gene segments encoding the amino and carboxyl termini. During lymphocyte ontogeny these mini-cassettes are inserted and stitched together to produce a functional VLR gene [9]. While there are no known recombination signal sequences to regulate the rearrangement, as required for the RAG-based Ig/TCR system, enzymes distantly related to activation-induced cytidine deaminase (AID) — CDA1 and CDA2 — are nonetheless expressed in lamprey lymphocytes and believed to orchestrate the rearrangement processes [10].

Over the next few years, studies concentrated on VLRB, which, like Ig, is found both on the lymphocyte cell surface and secreted into the plasma after lymphocyte stimulation, i.e. VLRB was found to be the lamprey equivalent of an 'antibody'. Immunization studies suggested that the VLRs were central to a T-cell-independent adaptive system, using antigen receptor cross-linking and pattern-recognition receptors for cellular activation leading to secretion [11]. This view was turned on its head when the second VLR locus, VLRA, was later studied. This receptor was found on another set of lymphocytes but never detected in the plasma [12]. Transcriptome analysis showed that the VLRB cells expressed gnathostome B-cell-specific genes (by chance VLRB had a suitable name!) like PAX5, and the VLRA cells expressed vertebrate T-cell-specific genes, such as Notch and IL-17. Boehm and colleagues then went on to show, again as a complete surprise, that the VLRA cells developed along the lining of the pharynx in lampreys, an area from which the thymus is derived in gnathostomes [13]. This so-called 'thymoid' expresses Notch ligands and chemokines important for T-cell differentiation in vertebrates, i.e. the simple transcriptional network underlying T-cell differentiation in gnathostomes (also uncovered by Boehm and colleagues [6]) is therefore also operable during differentiation of agnathan 'T cells'. This second wave of research into the VLRs totally astonished us, as the system seems replete with the equivalents of B cells, T cells, and a thymus!

In this review I will compare the lymphocyte lineages of jawed and jawless vertebrates based on recent work describing a new VLR, VLRC, which is expressed in cells having the properties of vertebrate $\gamma\delta$ T cells. In addition, I attempt to set the stage for further study of the lamprey adaptive immune system, with special attention to the relative complexity when compared with gnathostomes.

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This review is dedicated to the late Dr. Zeev Pancer, co-discoverer of the VLR system.

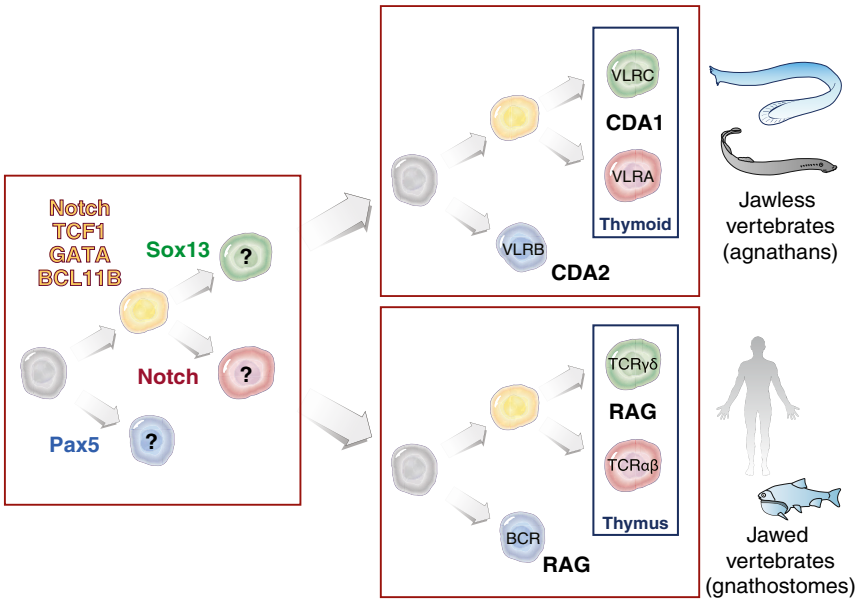

Figure 1. Lymphocyte lineages in jawless and jawed vertebrates (based in part on Supplemental Figure 6 in [31]).

Transcription factors required for T-cell precursors (yellow), γδ/VLRC T cells (green), αβ/VLRA T cells (red), and Ig/VLRB cells (blue) are shown on the left, in a hypothetical vertebrate ancestor. Cooper and colleagues [31] suggested that the transcriptional control of multiple lymphocyte lineages predated the emergence of any antigen receptors. Enzymes required for generation of the different antigen receptors are shown on the right.

A Third Lymphocyte Lineage, γδ T Cells, in Jawed Vertebrates

In the early 1980s a revolution occurred in immunology with the discovery of the TCR [14]. 'MHC restriction' of T-cell recognition was found about 10 years earlier [15], but it was a technical struggle to isolate the TCR. The β chain was cloned first and, while searching for the TCRα chain, another rearranging gene was discovered in T cells that was assumed to encode TCRα [16]. However, it quickly became clear that this was not the case, and instead a new TCR gene was discovered for which there was no biology to elucidate! A new heterodimeric TCR complex was found to be expressed on human tumor lines [17] and one of the chains was encoded by this new locus, the γ TCR. Several years later, the gene segments encoding the second TCR chain of this new complex — the δ chain — were found 'in the midst' of the TCRα locus [18]. We first thought that γδ T cells, like the αβ T cells, would also focus their recognition on MHC; however, over time a pioneer in the γδ field, Yueh-hsiu Chien, convinced us that, while γδ T cells can recognize some non-classical MHC class I ligands, by and large they recognize non-MHC ligands in an antibody-like manner [19].

γδ T cells were found to arise in the thymus in waves early in mouse development [20], with γδ T cells bearing particular gene signatures migrating to epithelia. The best studied of these are the skin-seeking dendritic γδ T cells (DETC) in mice, which emerge at day 16–17 during embryogenesis with invariant receptors and then self-renew for the life of the animal [21]. These cells are thought to act as 'first-line-of-defense' sentinels and are also important in wound healing. The ligand for this receptor is still unknown (although the cells are positively selected in the thymus on Skint, an Ig superfamily member [22]), but these cells have been the prime example of how γδ T cells function in an innate manner. In humans, the best-studied subset is the so-called Vγ2(9)/Vδ2 cells, found at high levels in the blood and recognizing metabolic pentose phosphate ligands expressed by microbes and self cells [23]. These γδ T cells also form a first line of defense against both viral and bacterial pathogens, and also can kill certain cancer cells. The molecule presenting these small ligands was unknown for 25 years, but recently another Ig superfamily molecule in the butyrophilin family has been implicated in this γδ T-cell recognition [24].

It should have been obvious early on, but while the 'first line of defense' argument for γδ T cells clearly holds water, it is also clear that they evolve rapidly, as even the major lineages of γδ T cells in mice and humans are quite different [21]. This is reminiscent of natural killer (NK) cells in mice and humans, as these cells do not even use receptors in the same gene family for their recognition events [25]. In addition, studies of non-placental vertebrates have shown that all other species (except bony fish) actually use IgVH genes in their TCRδ chains, most likely to recognize foreign antigens just like Ig, a result suggesting that Chien was quite prescient in her early predictions of γδ T-cell behavior. We and others (especially Miller and Parra from the University of New Mexico, who have performed the bulk of the work in this area) have suggested that γδ T cells not only recognize antigen with *bona fide* Ig elements, but that γδ T cells can perform adaptive functions as well [26,27]. This has been borne out in two recent studies in mice showing that responses to foreign and microbial antigens can be diverse and display memory, formerly the dominion of the αβ TCRs [28,29]. The bottom line is that, in contrast to αβ TCRs, γδ T cells are 'Nature's playthings', capable of being co-opted for a variety of innate and adaptive functions (except for MHC-restricted responses), depending on the life history of the organism in question (Figure 2). A corollary is that γδ T cells, even when adaptive, are activated more rapidly than αβ T cells [21].

A Third γδ-like Lineage of Lymphocytes in Lampreys?

Cooper and colleagues recently have examined a third VLR locus, VLRC [30,31]. Like the other VLRs, VLRC is expressed on its own subset of lymphocytes [31]. Like cells expressing VLRA, the VLRC-expressing cells do not secrete their receptors and develop in the thymoid. The VLRC-positive cells are found in much higher levels in skin epithelia compared with VLRA cells, similar to certain subsets of γδ T cells in mammals (although it should be emphasized that αβ T cells are also found in epithelia, especially in the gut and skin of mice/humans).

The VLRC epithelial-homing cells have a distinctive restricted repertoire compared with VLRA cells in the same tissues. This suggests, like the skin-seeking mouse γδ T cells mentioned above, that the lamprey cells might

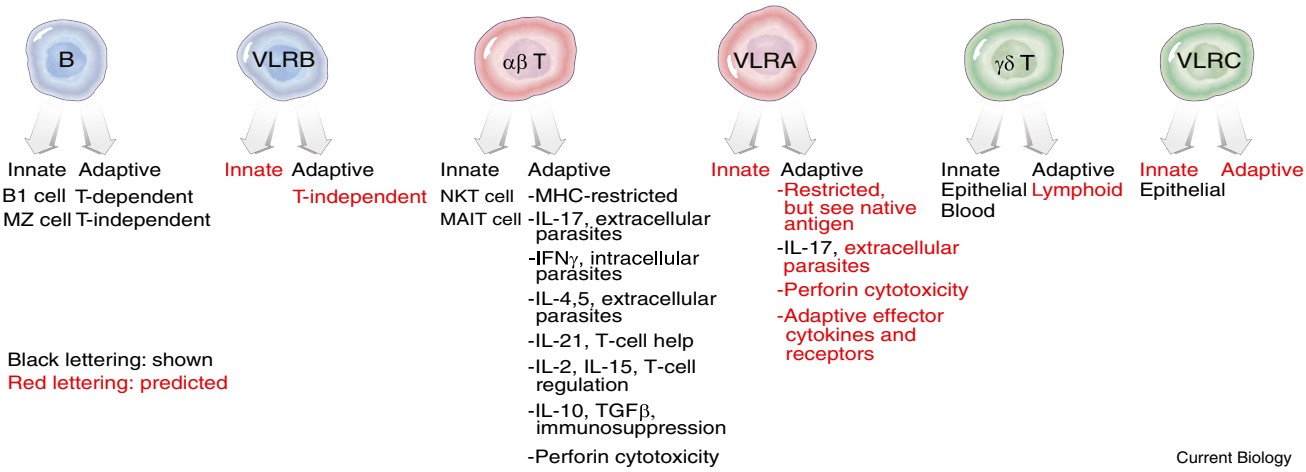

Figure 2. Comparisons of the phenotypes of the gnathostome (human and mouse, in this case) and agnathan (lamprey and hagfish) lymphocyte lineages.

Shown in black are known functions/cytokines/mechanisms. In red are my own predictions, based on discussion in the text.

recognize a conserved SOS ligand (i.e., a ligand induced by stress or infection, etc.). However, it should be emphasized that not all mammals even have receptor-restricted epithelial γδ T cells, so a direct link between mouse and lamprey is tenuous. While the transcriptome analysis showed conclusively that the VLRC is of the T-cell lineage, and at least one of the specific genes (SOX13) is a dedicated γδ marker in gnathostomes [32], the other VLRC-specific genes look to be specific either to the lamprey T-cell subset (e.g. TLR3) or to epithelial-homing T cells in general. Indeed, since γδ T cells evolve at a fast rate and there are several subsets — some epithelial-homing, some lymphoid tissue-homing — that produce different cytokines depending upon their selection in the thymus ([33] and see below), it may be difficult to definitively identify common expression patterns. The VLRA/VLRC rearrangement events in the thymoid suggest a tight regulation of the two gene families during lymphocyte development, not unlike the regulation of TCRs in the thymus. Classically, γ, δ, and β TCR genes were believed to rearrange simultaneously in developing gnathostome thymocytes, eventually resulting in different lineages based on successful rearrangement patterns, strength of antigen receptor signaling, and other factors [34,35]. As TCRs are heterodimers, there will always be developmental variations compared with the single-chain VLRs; nevertheless, similarities in the developmental progression of αβ and γδ T cells on the one hand and VLRC and VLRA cells on the other are eye-popping.

In summary, the lamprey data leave little doubt that there is a second agnathan T-cell lineage, beginning with a dedicated receptor and differentiation (and rearrangement) in the thymus equivalent. The transcriptome, repertoire, and epithelial-homing characteristics are less closely related to the γδ T cell lineage, but, since there is no consensus on a γδ T-cell transcriptome considering their rapid evolution in the gnathostomes, as well as their capacity to be either innate or adaptive, no one would anticipate a precise correlation between these cells in jawless and jawed vertebrate systems (Figure 2).

Based on this recent publication, and the study of the VLR system as a whole, the authors suggest that the αβ (VLRA), γδ (VLRC), and Ig (VLRB) lymphocyte lineages existed before the emergence of the antigen receptors (Figure 1), and the different antigen receptor families were co-opted in jawless and jawed vertebrates [31]. This is possible: as another example, NK cells have much in common with T cells regarding signaling and effector functions, but lack any somatically generated antigen receptor, and thus may have pre-dated cells with rearranging receptors. It is difficult to see what function a primordial 'B cell' might accomplish without an antigen receptor, as effector functions for B cells are found within the secreted antigen receptor, not the cell itself (true of VLRB as well, as shown in a recent study [36]). Perhaps the VLRB arose first, and indeed was used in a T-cell-independent system [11] (M. Kasahara, personal communication); indeed, since B cells from ectothermic vertebrates and mammalian B1 cells are capable of phagocytosis, this lineage may have retained primitive myeloid cell characteristics [37,38]. All in all, this is perhaps the first Big Bang accounting for adaptive immunity, and conserved early transcriptional networks coincided with the 'invention of lymphocytes' [39]. Certain leukocyte receptors that have been hypothesized to be primordial, based on their first appearance in early deuterostomes, may help identify the 'pre-antigen receptor' lineages [40].

Effector Functions and Immunoregulation

While the lymphocyte lineages seem to be conserved in all vertebrates, at first glance the range of chemokines and effector cytokines involved in immunity seem quite limited, based on the work on agnathan ESTs and the lamprey genome project (Table 1, Figure 2) [12,41]. However, caution must be embraced before we propose a second Big Bang of effectors in adaptive immunity arising in the gnathostomes. Recent work in which I was involved was mistaken in suggesting that the oldest extant gnathostomes, the cartilaginous fish, lacked many of the $CD4^+$ T-cell effectors and perhaps CD4 itself [42]. It turned out that several cytokines/receptors were not detected due to their low sequence similarity to higher vertebrate cytokines [43] and in fact sharks seem to have, if not a full-blown CD4 system, at least genes related to cytokines produced by all conventional $CD4^+$ T-cell lineages. So, based on our experience with shark cytokines/receptors, we should not underestimate the breadth of the

agnathan effector adaptive system. Conversely, we can be more confident that the agnathans truly lack MHC class I and II, immunoproteasome, etc., and thus the antigen presentation system is most likely a convergent one.

What are the effector functions of the lamprey T cells? When the dichotomy between VLRA and VLRB was uncovered, a model was proposed in which reciprocal interactions between T and B cells would result in T-cell help for B-cell stimulation, i.e. T cells express IL-17 and the IL-8 chemokine receptor (CXCR2), while B cells express the IL-17 receptor and the chemokine IL-8 [12]. This scenario may be true, but in my opinion it may be a minor T-cell function. In vertebrates, the function of IL-17 is much more important in direct defense against pathogens (induction of phagocytosis via stimulation of epithelia), than in acting as a helper factor for B cells. Based on the many pattern-recognition receptors expressed by lamprey B cells, perhaps the original idea that VLRB cells respond to antigen via their surface VLRB and pattern-recognition receptors is true, and the VLRA and VLRC cells may be more important for destruction of intracellular pathogens via cytotoxicity (although perforin has not been detected in lampreys to date [44]) and extracellular pathogens via phagocytosis (Figure 2). Finally, in addition to the well-known cytokines/chemokines that may have been overlooked in agnathans, as in sharks, there may be molecules that we do not yet recognize that perform similar or even unique functions.

Outlook for the Evolution of Adaptive Immunity

First and foremost, what do the lamprey T cells 'see'? In the likely absence of gnathostome MHC, is there some convergent molecule/system that presents antigen to VLRA or VLRC T cells? Molecular studies of VLRA and VLRB have shown that VLRA has a generally larger binding site, with one region that is relatively conserved and another that is quite diverse, suggesting that there may indeed be a 'restricting element' recognized by the conserved element, while the diverse region recognizes true antigen [45,46]. One study suggested that VLRA from an antigen-experienced adult lamprey could see native antigen with high affinity [45], suggesting that, if there is a restricting element, it associates with foreign antigen not as peptides but perhaps as whole antigen. One polymorphic molecule in hagfish, NICIR/ALA [47,48], was shown recently to be a candidate for one of these 'restricting elements.' Resolving this issue is the next Holy Grail in the field.

In the vertebrate thymus, αβ T cells undergo positive and negative selection on self-MHC molecules. With the discovery of the thymoid, the question is whether the same processes occur in jawless vertebrates. We all assume that negative selection will take place because an adaptive system must be made tolerant, but positive selection is a different problem. While the new candidate for MHC restriction (NICIR/ALA) is the immediate molecule of interest as a putative restricting element, other experiments can be performed straightaway. For example, the 'pre-repertoire' of VLRA- and VLRC-expressing cells in the thymoid can be compared with the repertoire of mature VLRA and VLRC T cells found in the periphery; if there are constraints imposed by 'MHC' restriction, one may find that a randomly-sized thymoid repertoire becomes shaped and constrained after 'thymoid selection.' Finally, as described above, mammalian γδ T cells generally are not MHC-restricted, but they can be selected by other thymic ligands [21]. Such selection bestows a different program of cytokine secretion compared with other γδ T cells, which are believed to form the adaptive pool of cells [33]. The study of antigen recognition by the VLRA and VLRC T cells could shed light on how antigen is detected by the mysterious adaptive γδ T cells, which are likely to see antigen on antigen-presenting cells in a non-MHC-restricted manner [19,27].

While it is most obvious for the γδ T cells, there are 'innate' and 'adaptive' lineages of αβ T cells (e.g. NKT cells) and B cells (e.g. B1 and marginal zone B cells) in gnathostomes. Will this be true of the agnathan lymphocyte lineages as well (Figure 2)?

In gnathostomes, RAG1 and RAG2 perform the rearrangement events in all antigen receptor loci. In this case

Table 1. Immune system characteristics in jawless and jawed vertebrates.

Property/molecule/cell	Jawless vertebrates (lamprey, hagfish)	Jawed vertebrates (e.g. humans, bony fish)
Adaptive immunity	+	+
Thymus	+ ('thymoid')	+
Spleen	–	+
Lymph nodes	–	+ (warm-blooded)
αβ T cells	+ (VLRA)	+ (αβ TCR)
γδT cells	+ (VLRC)	+ (γδ TCR)
NK cells	?	+ (many types of receptors)
Innate lymphoid cells (ILC)	?	+ (so far only found in mammals)
B cells	+ (VLRB)	+ (Ig)
Enzymes for GOD	+ (APOBEC, CDA1, CDA2)	+ (RAG)
Enzymes for affinity maturation	+? (APOBEC: CDA1, CDA2)	+ (APOBEC: AID)
MHC class I/II/β2m	–	+
Non-classical MHC class I	–	+
Immunoproteasome	–	+
Transporter associated with antigen processing (TAP)	+ (TAP-L)	+ (TAP-1/2/L)
Developmental transcription factors	TCF1, BCL11B, Notch, PAX5, SOX13, GATA	TCF1, BCL11B, Notch, PAX5, SOX13, GATA
Adaptive cytokines	IL-17 (others?)	IL-17, IFNγ, IL-2, IL-4, IL-5, IL-7, IL-10, IL-13, IL-15, IL-21 (and many more*)
β5T	–	+
AIRE	–	+
Costimulatory B7 family	+ (a few)	+ (many)
TNF family	+ (a few)	+ (many)
Alternative complement pathway	+	+
Classical complement pathway	–	+
Lectin complement pathway	+	+
Complement membrane attack complex	–	+

While the three lymphoid lineages and primary lymphoid tissues are present in both jawless and jawed vertebrates, MHC, secondary lymphoid tissues, and the plethora of adaptive cytokines/chemokines found in gnathostomes apparently are not present in agnathans.
GOD, generation of diversity for antigen receptors; β5T, proteasome enzyme specifically expressed by the thymic epithelium; AIRE, autoimmune regulator, a transcription factor that induces expression of tissue-specific genes by thymic medullary epithelium.

the particular Ig or TCR loci 'chosen' to rearrange are determined by accessibility of the different gene elements [49]. By contrast, agnathans seem to have dedicated two different cytidine deaminase family members, CDA1 and CDA2, to orchestrate rearrangement in T and B cells, respectively [10]. It will be interesting to determine which elements of the VLR loci are targeted by these different enzymes. Furthermore, there is evidence from one study that the VLRA cells might undergo affinity maturation during an immune response [45], suggesting that CDA1/CDA2 might be used both for repertoire generation and for mutation after activation of mature cells.

All gnathostomes have dedicated secondary lymphoid tissues, such as the spleen, in which B cells and T cells are segregated and adaptive immune responses are initiated [50]. Agnathans do not appear to have such tissues and seem to lack the chemokines/chemokine receptors important for the development of these tissues. Thus, how are adaptive responses initiated *in vivo*?

Could some lower chordates, such as amphioxus or *Ciona*, also have the precursor of the transcription-factor-driven development of lymphocytes, pre-dating the emergence of adaptive antigen receptors of any type? In mammals, cells called innate lymphoid cells share many of the properties of T cells, but have no somatically generated antigen receptors. Studies of early development of hematopoietic cells in lower chordates might reveal primordial features of the emergence of lymphocytes.

Conclusions

We have been amazed again and again by the discoveries made with the agnathan adaptive immune system, beginning with the report of the VLRs in 2004. A divergence of B and T cells, two lineages of T cells, as well as a thymus equivalent in lampreys were not envisaged based on 40 years of work in comparative immunology. I am sure that more surprises await us, the most anticipated being the likely convergent mechanism of antigen presentation.

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